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**Complement receptor 1 is the host erythrocyte receptor for *Plasmodium falciparum* PfRh4 invasion ligand**

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*Plasmodium falciparum* is responsible for the most severe form of malaria disease in humans, causing more than 1 million deaths each year. As an obligate intracellular parasite, *P. falciparum*'s ability to invade erythrocytes is essential for its survival within the human host. *P. falciparum* invades erythrocytes using multiple host receptor-parasite ligand interactions known as invasion pathways. Here we show that CR1 is the host erythrocyte receptor for PfRh4, a major *P. falciparum* ligand essential for sialic acid-independent invasion. PfRh4 and CR1 interact directly, with a  $K_d$  of 2.9  $\mu$ M. PfRh4 binding is strongly correlated with the CR1 level on the erythrocyte surface. Parasite invasion via sialic acid-independent pathways is reduced in low-CR1 erythrocytes due to limited availability of this receptor on the surface. Furthermore, soluble CR1 can competitively block binding of PfRh4 to the erythrocyte surface and specifically inhibit sialic acid-independent parasite invasion. These results demonstrate that CR1 is an erythrocyte receptor used by the parasite ligand PfRh4 for *P. falciparum* invasion.

malaria | red blood cell | merozoite | reticulocyte-binding-like homologue

Erythrocyte invasion is essential for the survival of *Plasmodium falciparum* within the human host. The merozoite forms of *P. falciparum* invade erythrocytes through a multistep process that involves initial contact with the erythrocyte, apical reorientation, and formation of a tight junction that moves progressively toward the posterior end of the parasite until host cell membrane fusion is complete (see ref. 1 for a review). These steps in invasion are dependent on specific interactions between multiple parasite invasion ligands and their respective human erythrocyte receptors, which have been defined as distinct invasion pathways.

In *P. falciparum*, two gene families encode important proteins used in invasion: the erythrocyte-binding-like antigens (EBAs: EBA-140/BAEBL, EBA-175, EBA-181/JESEBL, and EBL-1) and reticulocyte-binding-like homolog proteins (RBPs, or PfRh: PfRh1, PfRh2a, PfRh2b, PfRh4, and PfRh5) (2–5). During invasion, these ligands are localized at the apical tip of the merozoite and are able to bind erythrocytes. Invasion pathways have been identified by examining the entry of merozoites into erythrocytes that have deficient or mutant host receptors or that have been treated with enzymes that modify the properties or presence of erythrocyte surface proteins. The most common enzyme treatments involve neuraminidase, which removes sialic acid residues, and trypsin or chymotrypsin, which differentially cleave the peptide backbones of proteins. At present, only a handful of erythrocyte receptors that bind to *P. falciparum* invasion ligands have been identified, including glycophorin A for EBA-175, glycophorin B for EBL-1, and glycophorin C for EBA-140 (6–8). All three of these interactions are sensitive to neuraminidase treatment of erythrocytes and thus are involved in sialic acid-dependent invasion pathways. Complement receptor 1 (CR1) was recently identified as a receptor for the sialic acid-independent invasion pathways in multiple laboratory strains and wild isolates, although the parasite ligand with which it interacts has not yet been identified (9). CR1

also mediates rosetting through its interaction with PfEMP-1, a parasite-derived variant erythrocyte membrane protein (10). On the erythrocyte surface, CR1 is present as a ~190- to 280-kDa single-chain transmembrane glycoprotein bearing the Knops blood group (11).

PfRh4 is essential in the sialic acid-independent pathway as disruption of the gene in W2mef results in the inability of this strain to switch invasion pathways to allow invasion into neuraminidase-treated erythrocytes (12). Growth assays in the presence of anti-PfRh4 antibodies have shown that PfRh4 is the major ligand responsible for invasion via the sialic acid-independent pathways (50–80%, depending on the parasite strain used) (13). By activating PfRh4 expression, the parasite is able to switch receptor usage from sialic acid-dependent to sialic acid-independent pathways, thereby providing a mechanism for the parasite to invade via different pathways (12). Because parasite entry into erythrocytes is an essential component of the life cycle of *P. falciparum*, the use of multiple redundant invasion pathways and the ability to switch pathways through differential expression of parasite ligands provides the parasite with mechanisms to increase successful invasion in the face of host immune responses and erythrocyte receptor polymorphisms in malaria endemic regions (*SI Text*, [Fig. S1](#)).

Identifying the parasite ligand–host erythrocyte receptors used in invasion is a crucial step toward a complete understanding of the full repertoire of invasion pathways available to *P. falciparum*. Because PfRh4 is a key player in phenotypic variation of invasion and sialic acid-independent pathway, we sought to identify its host erythrocyte receptor. We observed that erythroid CR1 is susceptible to trypsin and chymotrypsin treatment, an enzyme profile consistent with that obtained for the abolishment of PfRh4 erythrocyte binding (13, 14). Here we show that PfRh4 binds to CR1 on the erythrocyte surface, and that this interaction mediates a functional sialic acid-independent pathway for *P. falciparum* invasion of human erythrocytes.

## Results

**PfRh4 Binds to CR1 on the Erythrocyte Surface.** To examine whether CR1 is a receptor for PfRh4, we incubated erythrocytes with anti-CR1 antibodies, then performed an erythrocyte-binding assay

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using culture supernatants containing *P. falciparum* invasion ligands released from merozoites (Fig. 1A). Using erythrocytes precoated with anti-CR1 antibodies, we found that PfRh4 erythrocyte binding was reduced, whereas EBA-175 binding was not perturbed (Fig. 1A). The addition of increasing amounts of anti-CR1 antibodies resulted in enhanced inhibition of PfRh4 erythrocyte binding, with >90% of binding blocked at an antibody dose of 0.0107 mg/mL (Fig. 1A). Preincubation of erythrocytes with glycophorin A/B or decay accelerating factor (DAF) antibodies did not affect PfRh4 binding, indicating that the inhibition observed with anti-CR1 antibodies is specific to PfRh4 (Fig. 1B).

We also tested the ability of soluble recombinant CR1 to inhibit binding of PfRh4 to the surface of human erythrocytes. Soluble CR1 (sCR1) contains the extracellular domain but lacks the transmembrane and cytoplasmic domains (15). Culture supernatants were preincubated with sCR1 before being added to erythrocyte-binding assays. Increasing amounts of sCR1 competitively blocked native PfRh4 binding to CR1 on erythrocytes, with >90% inhibition obtained at 0.04 mg/mL (Fig. 1C). Probing of the same binding eluates with EBA-175 antibodies resulted in no perturbation of EBA-175 erythrocyte binding, indicating that the inhibition is specific to PfRh4. Thus, PfRh4 binds to CR1 on the erythrocyte surface.

**Recombinant PfRh4 and CR1 Interact Directly.** To provide additional evidence of a PfRh4–CR1 interaction, we performed immunoprecipitation experiments using sCR1 and recombinant PfRh4, which is functional, as demonstrated by its ability to bind erythrocytes (Figs. S2 and S3) (13, 15). Using anti-PfRh4 monoclonal antibody 10C9, a complex containing both recombinant PfRh4 and sCR1 was immunoprecipitated (Fig. 2A). Given CR1's ability to bind immune complexes, we showed, as a control, that the 10C9 monoclonal antibody does not nonspecifically immunoprecipitate sCR1 (Fig. 2A). For an antibody control, we used anti-Rh4 monoclonal antibody 2E8, which does not recognize recombinant

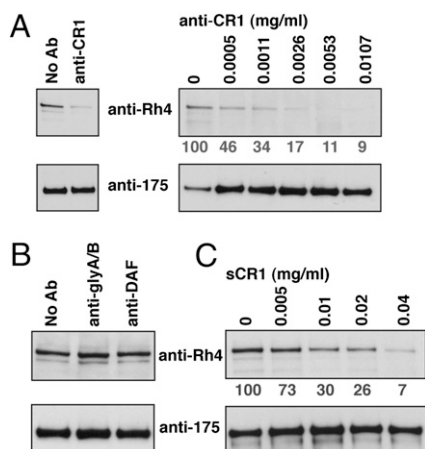
PfRh4; this did not immunoprecipitate the CR1–PfRh4 complex (Fig. S3B and C). Immunoprecipitation with an anti-CR1 antibody also isolated a complex containing recombinant PfRh4 and sCR1, but not another hexaHis-tagged control protein, further supporting the specificity of the PfRh4–CR1 interaction (Fig. 2B).

We also measured the CR1–PfRh4 interaction using an ELISA-based assay, in which sCR1 bound to immobilized recombinant PfRh4 but not to the control protein, and this binding increased with increasing concentrations of sCR1 (Fig. 2C and Fig. S3). This result was reinforced by surface plasmon resonance (SPR)-based studies (Fig. 2D). Soluble CR1, but not BSA, binds to recombinant PfRh4 that has been immobilized via amine coupling to a sensor chip. Measuring the binding of an sCR1 concentration series (at two different loadings on the chip of recombinant PfRh4) yielded a  $K_d$  of  $2.9 \pm 0.2 \mu\text{M}$  for the sCR1–PfRh4 interaction (Fig. 2D).

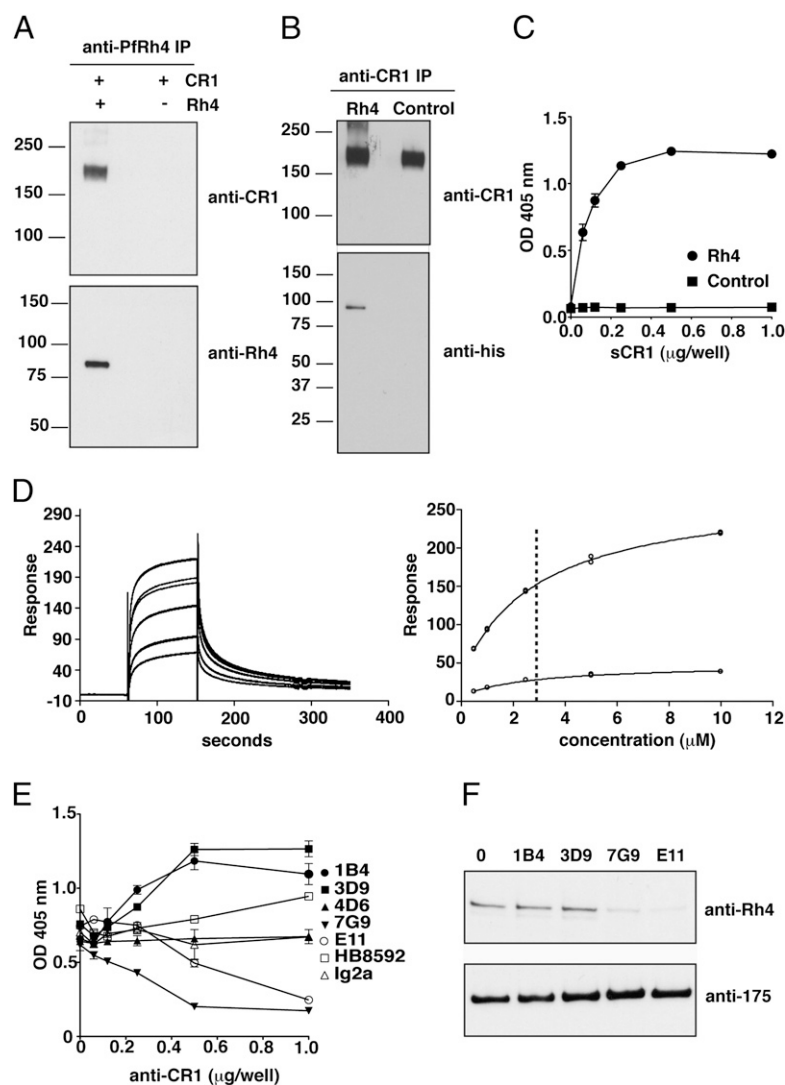
In an attempt to delineate the region within CR1 involved in PfRh4 binding, we measured the CR1–PfRh4 interaction in the presence of anti-CR1 monoclonal antibodies mapped to the extracellular domain of CR1 (Fig. S3) (16). In the most common isoform of CR1, the extracellular domain comprises 30 short consensus repeat modules (SCRs). Based on a high degree of internal homology, all except the last two carboxy terminal SCRs form larger units called long homologous repeats (LHRs), each of which is composed of seven SCRs. The subclass for the mouse monoclonal antibodies was IgG1 with the exception of 7G9, which was IgG2a. The addition of anti-CR1 monoclonal antibodies or Ig2a mouse isotype resulted in three distinct phenotypes for CR1–PfRh4 interaction in the ELISA-based assay. First, the addition of 4D6 (which recognizes SCRs 3, 10, and 17) or Ig2a mouse isotype did not affect this interaction. Second, the CR1–PfRh4 interaction was enhanced in the presence of 1B4, 3D9 (which also recognize SCRs 3, 10, and 17), and HB8592. Third, the CR1–PfRh4 interaction was disrupted by 7G9 and E11 monoclonal antibodies (Fig. 2E). These monoclonal antibodies recognize similar epitopes present at SCRs 5–7, 12–14, and 19–21. Although HB8592 recognizes the same class of epitopes as E11 (SCRs 5–7, 12–14, 19–21, and 26–28), it did not inhibit the interaction between sCR1 and PfRh4. This may be because E11 is able to immunoprecipitate LHR A and B, whereas HB8592 shows reactivity only to recombinant LHR D (16). Although this finding implicates one or more of SCRs 5–7, 12–14, and 19–21 of CR1 as the region involved in PfRh4 binding, the binding of antibodies to CR1 SCRs adjacent to (rather than within) ligand-binding sites has been reported to perturb function (16). Consistent with results for the inhibition of CR1–PfRh4 interaction, the addition of E11 and 7G9 produced a reduction in PfRh4 binding in standard erythrocyte-binding assays (Fig. 2F).

**Levels of PfRh4 Binding Were Strongly Correlated with CR1 Level on the Erythrocyte Surface.** CR1 levels on erythrocytes vary between individuals in the range of 50–1,200 molecules per cell. In Caucasian populations, single nucleotide polymorphism (SNP) within exon 22 in the CR1 gene is linked to high CR1 expression (H, high allele) or low CR1 expression (L, low allele) (17). Homozygous HH individuals have higher erythrocyte surface levels of CR1, homozygous LL individuals have <200 molecules per erythrocyte, and HL individuals having intermediate CR1 surface levels. To test whether differential expression of CR1 on erythrocytes correlated with PfRh4 binding, we analyzed the CR1 phenotype of blood samples from Australian residents. CR1 erythrocyte levels from these individuals showed an association with exon 22 genotyping (Fig. 3A). We observed markedly reduced native PfRh4 binding in erythrocytes from a LL individual compared with those from an HH individual in a standard erythrocyte-binding assay (Fig. 3B and Fig. S4D). We probed the same binding eluates with EBA-175 antibodies, but found no difference in EBA-175 binding.

To measure the level of PfRh4 erythrocyte binding in more blood samples, we developed a FACS-based assay for erythrocyte



**Fig. 1.** Anti-CR1 antibodies and sCR1 inhibit PfRh4 erythrocyte binding. (A) Anti-CR1 antibody (E11) inhibits PfRh4 erythrocyte binding (Left). Increasing concentrations of anti-CR1 monoclonal enhanced the reduction in PfRh4 erythrocyte binding (Right). Anti-CR1 monoclonal antibodies at 0–0.0107 mg/mL were incubated with erythrocytes before the addition of culture supernatants. (B) PfRh4 erythrocyte binding is not affected by preincubation of glycophorin A/B (glyA/B) or DAF monoclonal antibodies at final concentrations of 0.03 mg/mL. (C) Native PfRh4 binding to CR1 is inhibited by sCR1. Competitive binding assays with sCR1 were performed by incubating sCR1 with invasion supernatants at the stated final concentrations (0–0.04 mg/mL). In A and C, the gray numbers under each top panel represent the percentage of PfRh4 binding relative to the no-antibody lane or the no-sCR1 lane, respectively. In all panels, immunodetection of parasite proteins with anti-PfRh4 and anti-EBA-175 antibodies after erythrocyte binding is shown.



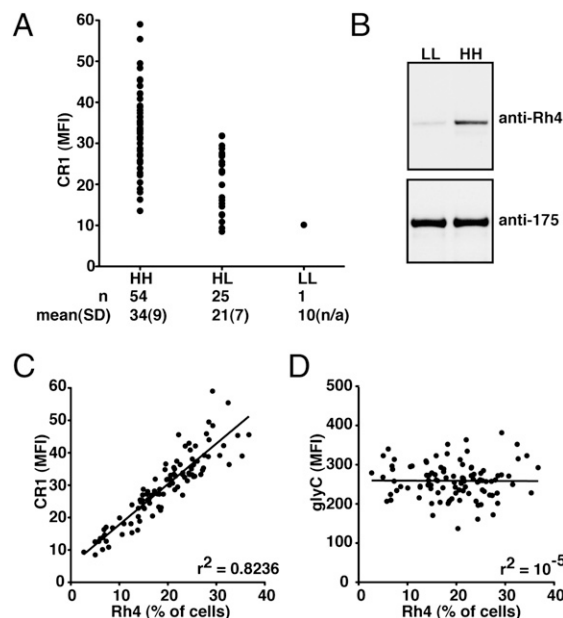
**Fig. 2.** Pfrh4 and CR1 directly interact. (A) Immunoprecipitation with anti-Pfrh4 monoclonal antibody 10C9 isolates a complex containing both recombinant Pfrh4 and sCR1. The anti-Pfrh4 monoclonal 10C9 does not immunoprecipitate sCR1 in the absence of recombinant Pfrh4. (B) Anti-CR1 monoclonal antibody HB8592 immunoprecipitates sCR1 and recombinant Pfrh4, but not another hexaHis-tagged protein (control). (C) An ELISA-based assay measuring the interaction between sCR1 and recombinant Pfrh4. Microtiter wells were coated with recombinant Pfrh4 or control hexaHis-tagged protein at 0.5  $\mu\text{g}/\text{well}$ . sCR1 was added at 0–1  $\mu\text{g}/\text{well}$ . Bound CR1 was detected with an anti-CR1 monoclonal HB8592. (D) Use of SPR to measure the dissociation constant ( $K_d$ ) of sCR1 for Pfrh4. (Left) Duplicate sensorgrams for a range of increasing sCR1 concentrations (0.5, 1.0, 2.5, 5.0, and 10  $\mu\text{M}$ , from bottom to top) flowed over a CM5 chip surface with a loading (via amine coupling) of 1,476 response units (RU) of recombinant Pfrh4. (Right) Plots of the RUs obtained versus sCR1 concentrations on two different flow cells, coupled with 408 RUs (lower curve) and 1476 RUs (upper curve) of recombinant Pfrh4. The dashed vertical line indicates the  $K_d$  fitted to both plots simultaneously. (E) Binding of sCR1 to recombinant Pfrh4 was inhibited by anti-CR1 monoclonal antibodies. Microtiter plates were coated with saturating concentration of recombinant Pfrh4 (5  $\mu\text{g}/\text{well}$ ). Anti-CR1 monoclonal antibodies and Ig2a mouse isotype at concentrations of 0–1  $\mu\text{g}/\text{well}$  were incubated with sCR1 (0.2  $\mu\text{g}/\text{well}$ ) before being added to the wells. Interaction between sCR1 and Pfrh4 was detected using anti-CR1 antibody 6B1 conjugated directly to HRP. 6B1 shares no similar epitopes with the other anti-CR1 antibodies. All ELISA experiments (C and E) were repeated, and similar results were obtained. In C and E, the y axis indicates absorbance measured at 405 nm. Error bars represent the range of duplicate readings. (F) Anti-CR1 antibodies inhibit native Pfrh4 erythrocyte binding in an epitope-specific manner. For erythrocyte-binding inhibition assays, the monoclonal antibodies were incubated with erythrocytes at 0.03 mg/mL before the culture supernatants were added. Lane 0 has no antibody added. Immunodetection of parasite proteins with anti-Pfrh4 and anti-EBA-175 antibodies after erythrocyte binding is shown.

binding using recombinant PfRh4 (Fig. S2) (18). The features of recombinant PfRh4 erythrocyte binding in a FACS-based assay are similar to those of standard binding assays in terms of enzyme sensitivity (Fig. S2). Analysis of 100 individual blood samples revealed a strong correlation between the percentage of erythrocytes that bound recombinant PfRh4 and the CR1 level on the erythrocyte surface ( $r^2 = 0.8236$ ; Fig. 3C and Fig. S2). In contrast, there was no significant correlation between recombinant PfRh4 binding and the level of surface glycophorin C ( $r^2 = 10^{-5}$ ; Fig. 3D). This indicates that the level of native or recombinant PfRh4

binding to erythrocytes is dependent on the amount of CR1 expressed on the erythrocyte.

**Sialic Acid-Independent Invasion Was Reduced in Low-CR1 Erythrocytes and Inhibited in the Presence of Soluble CR1.** To examine the role of CR1 in *P. falciparum* invasion, we screened 400 blood samples from nonoverlapping individuals to identify additional low-CR1 erythrocytes. We selected the 10 samples with the highest CR1 expression and the 10 samples with the lowest CR1 expression on the erythrocyte surface; in most cases, CR1 phe-

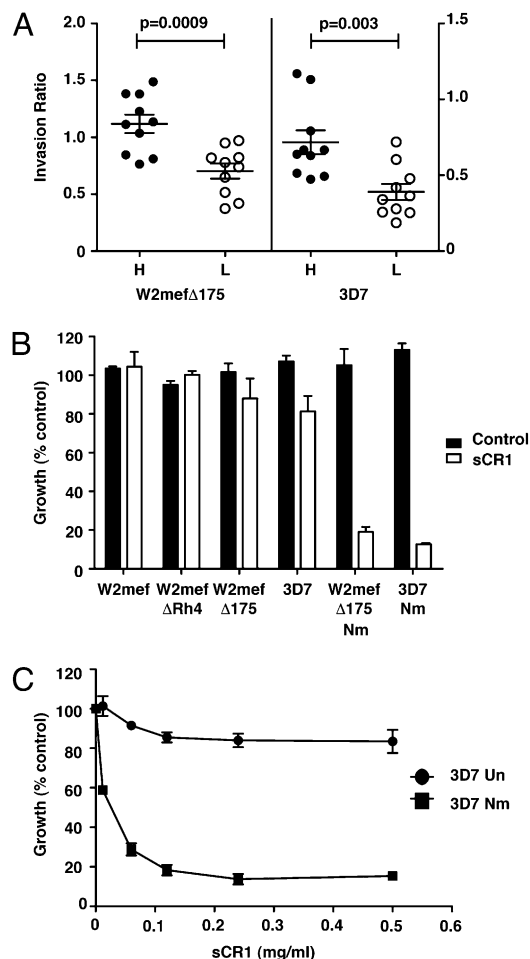




**Fig. 3.** The level of Pfrh4 binding correlates with CR1 expression on the erythrocyte surface. (A) Erythrocyte CR1 levels in relation to CR1 genotyping at exon 22 for 80 samples. Each point represents the average of MFI from duplicate readings. H, the high-CR1 allele; L, the low-CR1 allele; n, number of samples; n/a, not applicable. (B) Binding of native Pfrh4 to erythrocytes from LL and HH individuals. Immunodetection of parasite proteins with anti-Pfrh4 and anti-EBA-175 antibodies after erythrocyte binding is shown. (C) The percentage of recombinant Pfrh4-bound erythrocytes (x axis) correlates with the CR1 level on the erythrocyte surface (y axis). Recombinant Pfrh4 was added at 0.2 mg/mL to erythrocytes before proceeding with the FACS-based erythrocyte-binding assay, and binding was detected using anti-Pfrh4 monoclonal antibody. (D) Percentage of recombinant Pfrh4-bound erythrocytes (x axis) does not correlate with glycophorin C expression (y axis). In C and D,  $r^2$  is a measure of the goodness of fit of linear regression.

nototype was associated with exon 22 genotyping (Fig. S4). These erythrocyte samples were within normal ranges for blood indices and glucose-6-phosphate dehydrogenase (G6PD) activity and were wild type for other blood polymorphisms, including Gerbich, South Asian Ovalocytosis (SAO), and  $\alpha$ -thalassemia (Fig. S5). Although low-CR1 erythrocytes had higher levels of G6PD activity, this phenotype should not negatively affect parasite growth. Furthermore, none of the blood samples were microcytic or macrocytic. The level of recombinant Pfrh4 binding was again strongly associated with CR1 expression, whereas binding of recombinant Pfrh4 and EBA-175 did not correlate with levels of glycophorin C and CR1 expression, respectively (Figs. S4 and S5).

To examine the importance of CR1 as a receptor for the Pfrh4 invasion pathway, we evaluated the invasion of parasite strains W2mef $\Delta$ 175 and 3D7 into neuraminidase-treated erythrocytes (Fig. 4A). These strains have been shown to efficiently invade neuraminidase-treated erythrocytes, and Pfrh4 is known to be an essential invasion ligand for this sialic acid-independent pathway (12, 19). Thus, if CR1 is the receptor for Pfrh4, then this invasion pathway will be less efficient in the presence of neuraminidase-treated low-CR1 erythrocytes compared with invasion into neuraminidase-treated high-CR1 erythrocytes, due to the reduced availability of its receptor. Because both high-CR1 and low-CR1 erythrocytes showed variation in parasite growth rate, the efficiency of sialic acid-independent invasion in each sample (i.e., the invasion ratio) was calculated as a ratio of the percentage of parasitemia in neuraminidase-treated erythrocytes divided by the percentage of parasitemia in untreated erythrocytes using the same strain. These results are shown for high-CR1 erythrocytes in Fig. 4A. For both W2mef $\Delta$ 175 and 3D7, invasion



**Fig. 4.** Sialic acid-independent invasion is reduced in low-CR1 erythrocytes and inhibited in the presence of sCR1. (A) Parasite growth is reduced into neuraminidase-treated low-CR1 erythrocytes. The efficiency of sialic acid-independent invasion in each sample (i.e., the invasion ratio) was calculated as the ratio of the percentage of parasitemia in neuraminidase (Nm)-treated erythrocytes divided by the percentage of parasitemia in untreated erythrocytes using the same strain. These results are shown for invasion into high-CR1 erythrocytes (H, black circles) and low-CR1 erythrocytes (L, white circles) for both W2mef $\Delta$ 175 (left y axis) and 3D7 (right y axis). The mean  $\pm$  SEM invasion rates are shown.  $P$  values were determined using the unpaired Student  $t$  test. (B) The Pfrh4 invasion pathway is inhibited in the presence of sCR1. Parasite strains W2mef, W2mef $\Delta$ Rh4, W2mef $\Delta$ 175, and 3D7 were tested in growth assays into untreated or Nm-treated erythrocytes in the presence of 0.5 mg/mL of sCR1 (white bars) or control protein (black bars). (C) Inhibition of parasite growth by sCR1 is concentration-dependent. Invasion of 3D7 into untreated (un) or Nm-treated erythrocytes was measured in the presence of sCR1 at the stated 0–0.5 mg/mL final concentration. In B and C, growth is measured as the percent of noninhibitory PBS control, and error bars represent the SEM from two separate experiments (in triplicate).

into neuraminidase-treated low-CR1 erythrocytes was reduced compared with invasion into neuraminidase-treated high-CR1 erythrocytes ( $P = 0.0009$  and  $0.003$ , respectively). This decreased efficiency of the Pfrh4 invasion pathway results from the limited availability of CR1 on the erythrocyte surface.

We also examined parasite invasion rates in the presence of sCR1, which competitively inhibits binding of Pfrh4 to erythroid CR1 (Fig. 1C). We tested parasite strains W2mef, W2mef $\Delta$ Rh4, W2mef $\Delta$ 175, and 3D7 in invasion assays into untreated or neuraminidase-treated erythrocytes (Fig. 4B). Anti-Pfrh4 antibodies were shown to inhibit invasion of strains 3D7 (untreated), W2mef $\Delta$ 175 (neuraminidase-treated) and 3D7 (neuraminidase-treated) to 20%, 60%, and 80%,

respectively, but no inhibition was observed for W2mefΔRh4, W2mef, and W2mefΔ175 into untreated erythrocytes (13). This indicates that invasion of W2mefΔRh4, W2mef, and W2mefΔ175 into untreated erythrocytes is not dependent on PfRh4, whereas invasion of 3D7 (untreated), W2mefΔ175 (neuraminidase-treated), and 3D7 (neuraminidase-treated) is increasingly dependent on PfRh4 (Fig. S1). Parasite invasion into neuraminidase-treated erythrocytes by both 3D7 and W2mefΔ175 was greatly reduced in the presence of sCR1 (87% inhibition,  $P = 0.001$  and 81% inhibition,  $P = 0.010$ , respectively) (Fig. 4B and Fig. S4), but not in the presence of control protein. This inhibition of parasite invasion could be titrated, with decreasing amounts of sCR1 resulting in less invasion inhibition (Fig. 4C). Invasion of 3D7 into untreated erythrocytes also was slightly reduced, consistent with results obtained with anti-PfRh4 antibodies, although the difference was not statistically significant (20% inhibition;  $P = 0.09$ ). As controls, we observed no significant difference in parasite invasion in the presence of sCR1 into untreated erythrocytes with W2mef, W2mefΔRh4, and W2mefΔ175, strains that do not rely on PfRh4 as an invasion ligand ( $P = 0.913$ ,  $0.206$ , and  $0.352$ , respectively). These results demonstrate that CR1 is the host erythrocyte receptor used by PfRh4, and that this pathway functions directly in *P. falciparum* invasion.

## Discussion

Invasion of *P. falciparum* into human erythrocytes requires specific ligand–receptor interactions. The PfRh family of proteins is a group of key ligands important to invasion by direct binding to the erythrocyte. Up to now, no receptors have been identified, however. Here we show that CR1 serves as a receptor for *P. falciparum* invasion via direct binding of the parasite ligand PfRh4. The level of PfRh4 binding to erythrocytes is strongly correlated with the amount of CR1 molecules on the erythrocyte surfaces. In the presence of sCR1, low inhibition of parasite invasion into untreated erythrocytes was observed in sialic acid-independent strains 3D7 and W2mefΔ175. This is related to the redundancy of EBA and PfRh proteins, so that inhibition of one pathway is compensated for by the function of others. Treatment of erythrocytes with neuraminidase resulted in a blockage of invasion by such ligands as EBA-175, EBA-181, EBA-140, and PfRh1, which require sialic acid-containing receptors. As a result, parasite invasion into neuraminidase-treated erythrocytes was increasingly reliant on the PfRh4–CR1 pathway, as demonstrated by the strong inhibition of invasion by the addition of sCR1. This result is consistent with data reported by Spadafora et al. (9) showing that the addition of sCR1 inhibited parasite invasion into neuraminidase-treated erythrocytes in sialic acid-independent strains, such as 7G8 and 3D7. sCR1 was not able to inhibit invasion in strains that either lack PfRh4 expression (W2mef) or possess a genetic knockout of the PfRh4 gene (W2mefΔRh4), providing convincing evidence that the reduction in invasion was due to the sCR1–PfRh4 interaction. We also show that binding of PfRh4 to erythrocytes, as well as PfRh4–CR1 interactions, can be blocked by the presence of sCR1 or anti-CR1 monoclonal antibodies, thus providing a molecular basis for the inhibition of invasion. These results indicate that the PfRh4–CR1 interaction is a functional sialic acid-independent invasion pathway for the entry of *P. falciparum* into human erythrocytes. Although neuraminidase-treated erythrocytes do not exist in the field, this in vitro situation mimics situations in which host immune responses selectively block the function of specific parasite invasion pathways or erythrocyte polymorphisms in surface proteins result in a limited receptor repertoire.

CR1 has been shown to mediate rosetting through its interaction with PfEMP-1, a parasite-derived variant erythrocyte membrane virulence protein (10). Rosetting occurs when infected erythrocytes adhere to uninfected erythrocytes, resulting in “clumps.” The formation of rosettes can be disrupted by the addition of sCR1 and is reduced in the presence of low-CR1 ery-

throcytes (10). The CR1 region required for rosetting is SCR5 10 and 17. Anti-CR1 monoclonal antibodies that map to those regions (1B4, 3D9, and 4D6) do not inhibit either PfRh4 erythrocyte binding or the CR1–PfRh4 interaction, suggesting that the regions of binding to PfEMP-1 and PfRh4 might be distinct sites within CR1 (20). However, anti-CR1 monoclonal antibody J3B11 has been shown to inhibit parasite invasion into neuraminidase-treated erythrocytes and also to interfere with PfEMP-1 binding (9, 20). A major caveat with all of these experiments is that anti-CR1 monoclonal antibodies that recognize the same epitopes show contrasting results in terms of inhibition of rosetting, PfRh4 erythrocyte binding, and invasion assays (9, 20). In addition, it has been shown that binding of anti-CR1 antibodies to SCR5 adjacent to (rather than within) ligand-binding sites can perturb function (16). Finer epitope mapping of the anti-CR1 monoclonal antibodies and further studies using functional protein domains of CR1 will help elucidate the precise location within CR1 that binds PfRh4. Of note, *P. falciparum* uses the same host erythrocyte receptor for two distinct functions important for the virulence and survival of the parasite, which probably exploits the essential nature of CR1 on erythrocytes, given the absence of CR1 null erythrocytes in the human population.

Field isolates from India, Gambia, Brazil, Tanzania, and Kenya use many different invasion pathways, as demonstrated by the ability of these field parasites to invade a wide variety of enzyme-treated erythrocytes (21–25). Interestingly, a majority of Kenyan *P. falciparum* isolates invade via a neuraminidase-resistant, trypsin-sensitive, and chymotrypsin-sensitive pathway—an enzyme profile identical to that observed for PfRh4 erythrocyte binding (19 out of 31 isolates) (22). It is highly likely that PfRh4 is involved in this invasion pathway, given the fact that no other PfRh or EBA has a comparable enzyme profile. In support of this possibility, Spadafora et al. (9) reported the inhibition of invasion of three clinical isolates into both intact and neuraminidase-treated erythrocytes by the addition of sCR1. Determining the prevalence of the CR1–PfRh4 pathway in field isolates would require growth assays on more samples in the presence of inhibitory anti-PfRh4 antibodies or with the addition of sCR1 (13).

To prevent invasion, the human population relies on two protective mechanisms: a polymorphic erythrocyte surface and antibody responses to block parasite ligand function. Blood polymorphisms prevalent in malaria-endemic regions, such as Gerbich negativity in Papua New Guinea (PNG), could result in less-efficient invasion via EBA-140 (26). Human sera from malaria-exposed individuals contain antibodies to *P. falciparum* ligands and inhibit invasion in both sialic acid-dependent and –independent pathways (27). Thus, receptor polymorphisms and host immune responses against individual ligands might lead to inhibition of particular invasion pathways. The population of Madang, PNG, has the lowest CR1 levels reported to date (28). Our results indicate that the PfRh4–CR1 invasion pathway was less effective in the presence of low-CR1 erythrocytes, most likely due to limited receptor availability. However, studies in PNG have found no evidence for differences in parasite densities among HH, HL and LL genotypes for CR1, although the HL genotype is significantly protected from severe malaria (29). The lack of correlation between protection against severe malaria and reduced parasitemia has been observed for other blood polymorphisms, including  $\alpha$ -thalassemia and SAO, and remains a confounding dilemma in the field (30, 31). In terms of CR1 polymorphisms, the lack of measurable differences in parasite densities is likely due to the fact that HH, HL, and LL erythrocytes from PNG individuals all have low CR1 levels (<400 molecules per cell) compared with Caucasian individuals. The importance of PfRh4 as an invasion ligand in the PNG population is indicated by the strong reactivity to PfRh4 of sera from immune individuals (13). To date, no study has investigated the invasion phenotypes of PNG field isolates, and further work is needed to determine the relevance of these protective mechanisms.

Here we show that CR1 is a receptor used for *P. falciparum* invasion into erythrocytes. CR1 belongs to the regulator of complement activation (RCA) protein family and has been characterized as a negative regulator of complement activation. Several pathogens are known to bind to RCA proteins as a means to facilitate entry into host cells or to down-regulate complement activation (reviewed in ref. 32). RCA proteins, such as DAF, are present on erythrocytes and also may be involved in *P. falciparum* invasion.

## Materials and Methods

See *SI Materials and Methods* for more detailed information.

**Erythrocyte-Binding Assays.** Erythrocyte-binding assays and enzymatic treatment of erythrocytes were performed as described previously (13). For the antibody inhibition of erythrocyte binding, anti-CR1, anti-glycophorin A/B monoclonal, and anti-DAF monoclonal antibodies were preincubated with erythrocytes before the addition of culture supernatants. Competitive binding assays with sCR1 were performed by incubating sCR1 with culture supernatants before proceeding with standard erythrocyte-binding assays. The FACS-based erythrocyte-binding assay for *P. falciparum* is a modification of the protocol used for Duffy antigen binding (18).

**In Vitro Immunoprecipitation.** Recombinant proteins used in immunoprecipitations were incubated together in T-NET buffer [1% Triton X-100, 150 mM NaCl, 10 mM EDTA, and 50 mM Tris (pH 7.4)]. Immunoprecipitation was performed using anti-CR1 (HB8592) or anti-PfRh4 (10C9 and 2E8) monoclonal antibody coupled to Protein G/A Sepharose beads and then eluted in glycine elution buffer. Eluted proteins were resuspended in nonreducing sample buffer, run on SDS/PAGE gels, and evaluated by immunoblot analysis.

**ELISA.** Microtiter plates were coated with recombinant fusion protein, blocked, and incubated with ligand. Protein interactions were detected using a specific monoclonal antibody and an HRP-conjugated secondary antibody.

Azino-bis-3-ethylthiazoline-6-sulfonic acid was used to detect HRP activity, and optical density was measured at 405 nm.

**SPR.** Binding of sCR1 for PfRh4 was monitored by SPR using the Biacore T100 system (GE Healthcare). The sensor chip surfaces were prepared by immobilizing recombinant PfRh4 at different loading densities on two of the four flow cells of a Biacore Series 5 carboxymethylated dextran (CM5) sensor chip, using standard amine coupling. Experiments were performed at 25 °C at and a flow rate of 30  $\mu$ L/minute. Duplicate injections of sCR1 samples in 10 mM Hepes-buffered 150 mM saline with 3 mM EDTA and 0.005% (vol/vol) polysorbate 20 (pH 7.4) were performed at the concentrations indicated in Fig. 2. Data were processed using Biacore T100 evaluation software version 2.0. Dissociation constants were calculated by fitting steady-state binding levels derived from the background-subtracted traces to a one-to-one binding steady-state model.

**Growth Assays.** Parasite growth assays were performed over two cycles of parasite growth as described previously (27).

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